Biogeographical patterns of legume-nodulating *Burkholderia*: from African Fynbos to continental scales

Benny Lemaire[#1,2], Samson Chimphango[1], Charles Stirton[1], Suhail Rafudeen[3], Olivier Honnay[2], Erik Smets[2,4], Wen-Ming Chen[5,] Janet Sprent[6], Euan K. James[7], A. Muthama Muasya[1]

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[1]*Department of Biological Sciences, University of Cape Town, Private Bag X3, Rondebosch 7701, Cape Town, South Africa*

[2]*Plant Conservation and Population Biology, KU Leuven, Kasteelpark Arenberg 31, PO Box 2435, 3001 Heverlee, Belgium*

[3]*Department of Molecular and Cell Biology, University Cape Town, Cape Town, South Africa*

[4]*Naturalis Biodiversity Center, Leiden University, 2300 RA Leiden, The Netherlands*

[5]*Laboratory of Microbiology, Department of Seafood Science, National Kaohsiung Marine University, No. 142, Hai-Chuan Rd. Nan-Tzu, Kaohsiung City 811, Taiwan*

[6]*Division of Plant Sciences, University of Dundee at JHI, Dundee,DD2 5DA UK*

[7]*The James Hutton Institute, Invergowrie, Dundee, DD2 5DA, UK*

#Corresponding author
Department of Biological Sciences
University of Cape Town, Private Bag X3
Rondebosch 7701
South Africa
E-mail address: benny.lemaire@uct.ac.za

Plant Conservation and Population Biology
KU Leuven, Kasteelpark Arenberg 31, box 02435
3001 Heverlee
Belgium
TEL. + 32 16 32 86 36 FAX + 32 16 32 19 68
E-mail address: benny.lemaire@bio.kuleuven.be

Abstract

Rhizobia of the genus *Burkholderia* have large-scale distribution ranges, and are usually associated with South African papilionoid and South American mimosoid legumes, yet little is known about their genetic structuring at either local or global geographical scales. To understand variation at different spatial scales, from individual legumes in the Fynbos (South Africa) to a global context, we conducted analyses of chromosomal (16S rRNA, *recA*) and symbiosis (*nifH, nodA, nodC*) gene sequences. We showed that the global diversity of nodulation genes is generally grouped according to the South African papilionoid or South American mimosoid subfamilies, whereas chromosomal sequence data were unrelated to biogeography. While nodulation genes are structured on a continental scale, a geographical or host specific distribution pattern was not detected in the Fynbos region. In host range experiments, symbiotic promiscuity of *Burkholderia tuberum* STM678^T and *B. phymatum* STM815^T was discovered in selected Fynbos species. Finally, a greenhouse experiment was undertaken to assess the ability of mimosoid (*Mimosa pudica*) and papilionoid (*Dipogon lignosus, Indigofera filifolia, Macroptilium atropurpureum* and *Podalyria calyptrata*) species to nodulate in South African (Fynbos) and Malawian (Savanna) soils. While the *Burkholderia*-philous Fynbos legumes (*D. lignosus*, *I. filifolia* and *P. calyptrata*) only nodulated in their native soils, the invasive neotropical species *M. pudica* did not develop nodules in the African soils. The Fynbos soil, notably rich in *Burkholderia*, seems to retain nodulation genes compatible with the local papilionoid legume flora, but is incapable of nodulating mimosoid legumes which have their center of diversity in the South American continent.

Importance section

This study is the most comprehensive phylogenetic assessment of root-nodulating *Burkholderia* and investigates biogeographic and host-related patterns of the legume-rhizobial symbiosis in the South African Fynbos biome, as well as at global scales, including native species from the South American Caatinga and Cerrado biomes. While a global investigation of the rhizobial diversity revealed distinct nodulation and nitrogen fixation genes among South African and South American legumes, regionally distributed species in the Cape region were unrelated to geographical and host factors.

Introduction

Microorganisms have been observed to vary in distribution, diversity and species composition across spatial scales (1), challenging the long-held perception of a microbial cosmopolitism driven by their high dispersal capacities (2). Although microorganisms can disperse over lengthy distances, dispersal limitations have revealed spatially isolated microbial populations over multiple spatial scales (1,3-5). For rhizobia (both alpha- and beta-subclasses of the Proteobacteria), similar geographical distribution patterns have been detected in different bacterial groups and over various spatial scales, showing a geographical structure preserved in phylogenies of both chromosomal and nodulation genes (6-11).

Root-nodulating species of the genus *Burkholderia* (Betaproteobacteria), have been described from different regions in the world, including parts of the Americas, Africa, Asia and Australasia. The highest level of diversity has been reported from the South American Cerrado/Caatinga and South African Fynbos biomes (12), together with Asian and Australian/New Zealand representatives so far described exclusively from non-native invasive species, such as the weeds *Mimosa diplotricha*, *M. pigra*, *M. pudica* (13-18) and *Dipogon lignosus* (49,79). *Burkholderia* species isolated from native legumes from neotropical and African regions, which are dominated by distinct legume floras (South American Mimosoideae versus South African Papilionoideae), differ genetically in their nodulation genes (12,19), suggesting that the legume host is shaping symbiotic diversity and that the biogeography of rhizobia is linked to the distribution of compatible legume hosts ((20) and references therein). Despite many local surveys of *Burkholderia* interactions with papilionoids and mimosoids across the globe, our knowledge of the global distribution pattern is still fragmented and a spatial survey of the genus *Burkholderia* has never been conducted in a global context and across biomes.

In South Africa, *Burkholderia* symbionts are widespread and associated with diverse lineages of the tribes Crotalarieae (21-23,26), Hypocalypteae (24,25), Indigofereae (26), Phaseoleae (26,27,28) and Podalyrieae (24,26,29,30), indicating that the South African soils are an important reservoir for nodulating *Burkholderia*, and thus this needs to be explored further for new candidate species. With the exception of *B. phymatum* strains nodulating the non-native crop species *Phaseolus vulgaris* (common bean) in Moroccan soils (31), the legume-*Burkholderia* symbiosis in Africa has only been reported in a range of sites within the Fynbos region, supporting the idea of the Cape region as an exclusive biodiversity hotspot for the symbiosis (12).

The general aim of the present study is to provide novel insights into the biogeography of *Burkholderia* and to elucidate the extent to which it exhibits a geographical pattern in relation to the distribution of its hosts. Because lineages vary in distribution and diversity over various spatial scales, and spatial factors play a significant role in shaping microbial communities, it is clear that geographical patterning should be analyzed across multiple spatial scales (from local to broad geographical regions). We took advantage of the large record of root-nodulating *Burkholderia* established since the first reports of its nodulation ability ((12) and references therein), supplemented with new sequence data of Fynbos *Burkholderia*. Available sequence data for chromosomal 16S rRNA and the symbiosis-related *nodA*, *nodC* and *nifH* genes were analyzed in a world-wide perspective to assess geographic patterns at a continental scale, as well as the host specific interactions with the legume subfamilies Mimosoideae and Papilionoideae.

The diversity, geographic distribution and host associations were further investigated at a regional scale in the South African (Cape) Fynbos biome. The *Burkholderia* symbionts from five Cape legume tribes and 11 genera of the Papilionoideae were investigated by

phylogenetic analyses of two chromosomal genes (16S rRNA and *recA*) and one nodulation gene (*nodA*) in relation to their geography and host phylogeny.

We hypothesize that the *Burkholderia* symbionts of native and invasive legume species reported from Africa, America, Asia and Australasia exhibit a geographical distribution pattern with continents having their own subset of symbionts. We also expect a geographical effect on the genetic variation of rhizobia at a regional scale within the Fynbos. Our specific objectives were (1) to determine and compare the *Burkholderia* types for housekeeping and symbiosis loci recorded from mimosoids and papilionoids reported from four different continents; (2) to investigate the distribution pattern of *Burkholderia* and its host-associations within the Fynbos biome, using field-collected nodules of indigenous papilionoids; (3) to investigate the ability of South African papilionoid legume species (*Indigofera filifolia*, *Dipogon lignosus*, *Podalyria calyptrata*, *Psoralea pinnata*) and the South American species *Mimosa pudica* (subfamily Mimosoideae) to form nodules in South African (Fynbos) and Malawian (Savanna) soil; and (4) to test and evaluate the host range of the *Burkholderia tuberum* STM678^T and *B. phymatum* STM815^T type strains on selected Fynbos species, which are known to exhibit different host affinities as dictated by their genetically distinct nodulation genes. We expect that the tested papilionoid legumes from the Fynbos are exclusively nodulated by the common and native symbiont *B. tuberum* STM678^T.

Material and Methods

Burkholderia *datasets and OTU-based analyses*

Analyses of Operational Taxonomic Units (OTUs) were used to cluster the 16S rRNA sequence data. A large 16S rRNA data set was constructed, comprising 1121 sequences and 75 validly named *Burkholderia* species with multiple accessions per species. Sequences were aligned with available bacterial reference sequences via the Ribosomal Database Project (RDP pyrosequencing pipeline; http://pyro.cme.msu.edu). An uncorrected pairwise distance matrix was calculated and the number of OTUs and rarefaction curves at various cut-off values (0.030 to 0.010) were calculated in Mothur v.1.31.2 (33).

Four other *Burkholderia* datasets were obtained from available 16S rRNA (365 sequences), *nifH* (246 sequences), *nodA* (152 sequences) and *nodC* (199 sequences), assigning all rhizobia to four geographical regions (Africa, America, Asia and Australasia and two legume subfamilies (Papilionoideae and Mimosoideae). The alignments were created with Muscle (32) using Geneious v.5.1.7 (http://www.geneious.com). The diversity of 16S rRNA sequences was clustered into OTUs, using the previous estimated cut-off value to delineate taxonomic identities at species level. For the data sets of *nifH*, *nodA* and *nodC*, we applied a similar conservative similarity cut-off value in order to classify genetic groups of the more variable symbiosis genes. Unique and shared types among different continents and subfamilies were identified in Mothur.

Alignments for the NeighborNet analyses were compiled based on the previous 16S rRNA, *nifH*, *nodA* and *nodC* rhizobial datasets: one sequence representative per sequence cluster was manually selected from the original alignments and these were imported into SplitsTree v.4.12.8 (34) to display the phylogenetic relatedness among the clusters as a NeighborNet network (35), using the most complex model of nucleotide substitution (GTR) available. Bootstrap confidence values were generated using 1,000 permutations.

Nodule sampling, DNA extraction, amplification, cloning and sequencing to identify Fynbos rhizobia

We investigated 20 root nodulated Fynbos species in this study, representing various localities (Fig. 1) and diverse host legumes (five legume tribes and 11 genera). Voucher information and GenBank accession numbers are listed in Table S2 and the geographical localities are shown in Figure 1. Nodules were collected in the field from a broad geographical range at different localities, covering diverse soil types ranging from limestone substrate (De Hoop Nature Reserve, Still Bay), granite substrate (Paarl Mountain Nature Reserve), sandstone mountain slopes (Bainskloof Nature Reserve) to coastal deep sand (Cape Point Nature Reserve). Three to five nodules were removed from each host plant for isolation of rhizobia.

Rhizobia were identified by both standard culturing techniques (36) and direct genomic DNA extraction from nodules. The latter method enabled the unequivocal assessment of the intranodular endophyte diversity, including unculturable endophytes that can be masked using culturing-based techniques due to the selective effects of growth media and an incomplete sampling of colony morphotypes. For the standard culturing technique, rhizobia were isolated on yeast extract mannitol agar (YEMA) from a single bacterial colony type, following standard procedures (36). Pure rhizobial cultures from single colonies were stored at -80°C in YEM broth containing 20% glycerol. Total DNA of the rhizobial cultures was obtained by the following thermal cell lysis procedure: A loopful of bacterial culture was suspended in 20 µl lysis buffer (10% SDS, 1M NaOH) followed by incubation for 15 minutes at 95°C. The lysate was centrifuged at 10,000 g for 45 s and 180 µl of sterile water was added. The DNA extract was centrifuged for another 5 minutes at 10,000 g at 4°C and preserved at -20°C. For the direct DNA extraction from root nodules, genomic DNA of

surface-sterilized nodules was obtained using the E.Z.N.A.TM HP Plant DNA Mini Kit (Omega bio-tek) as per manufacturer's instructions.

PCR amplification of 16S rRNA used universal bacterial primers (27f and 1492r) as previously described (37). Amplicons of nearly complete 16S rRNA were sequenced and subjected to BLAST analyses on GenBank as a first identification tool. Amplification of the *recA* housekeeping gene and the *nodA* nodulation gene was carried out with the primers *recA*-63F, *recA*-504R, *nodA*-1F, *nodA*-2R and PCR parameters as described by Gaunt *et al.* (38) and Haukka *et al.* (39). Amplification of the *nodC* nodulation gene was carried out for selected Fynbos isolates, using the primers *nodC*-540 and *nodC*-1160. All primer sequences are listed in Table S3.

Amplified 16S rRNA products from total genomic DNA extractions of the nodules were cloned into a pGEM-T vector (Promega), according to the manufacturer's instructions, and transformed into JM109 *E. coli* by heat shock (40). Purified plasmids and all PCR products were sent to Macrogen for sequencing (Macrogen Inc, Seoul, Korea). Sequencing primers for 16S rRNA, *recA* and *nodA* were the same as for the initial PCR.

Authentication of cultured rhizobia from field nodules

Nodulation capabilities of isolates from field nodules were tested on siratro (*Macroptilium atropurpureum*) (36). Table S2 lists the authenticated isolates in this study together with previously tested strains (26). Rhizobial isolates from nodules of legume species (*Dipogon lignosus*, *Indigofera filifolia*, *Podalyria calyptrata* and *Psoralea pinnata*) grown in the greenhouse were authenticated on their respective host. Nodulation (three replicates) was assessed by either inoculating seedlings with a rhizobial culture (OD_{600}) or leaving them uninoculated as negative controls. Authentication was confirmed if isolates nodulated the

roots of inoculated plants from all replicated pots, and the uninoculated plants remained nodule-free.

Phylogenetic analyses of the 16S rRNA, recA *and* nodA *sequence data*

Sequence reads were assembled and sequence alignments were created with Muscle (32) using Geneious v.5.1.7 (http://www.geneious.com). For the combined phylogeny of 16S rRNA and *recA*, missing sequences due to the lack of amplification were treated as missing data. Phylogenetic relationships were conducted using Bayesian Inference (BI) and Maximum Likelihood (ML) optimality criteria. Bayesian analyses were carried out in MrBayes v.3.1 (41) after determining the appropriate model of evolution with MrModeltest v.3.06 (42) under the Akaike information criterion. Modeltest selected for the 16S rRNA, *recA* and *nodA* datasets the GTR+I+G model. Four Markov Chains were run simultaneously for four million generations, sampling every 100 generations. The initial 25% of trees were regarded as "burnin" and discarded. Convergence of the chains was checked using Tracer v.1.4 (43). ML analyses were performed using RAxML-VI-HPC v.7.0.4 (44). A total of 100 RAxML searches were conducted, relying on the GTR-GAMMA model of evolution. Support values were estimated using a multi-parametric bootstrap resampling with 1000 pseudo-replicates.

Geographic distances among Fynbos representatives

The genetic variation of rhizobia across spatial scales in the Fynbos was calculated on both chromosomal (*recA*) and nodulation (*nodA*) data. Genetic distance matrices for both sets of genes was constructed including our Fynbos isolates and supplemented with previously described rhizobial strains (see Fig. 1). The *recA* and *nodA* datasets comprised 134 and 128 sequences, respectively, covering genera of the tribes Podalyrieae (*Amphithalea*, *Cyclopia*,

Podalyria, *Virgilia*), Crotalarieae (*Aspalathus*, *Crotalaria*, *Rafnia*), Hypocalypteae (*Hypocalyptus*), Phaseoleae (*Bolusafra*, *Dipogon*) and Indigofereae (*Indigofera*). Genetic variation of all pairs of isolates was linked with a geographic distance matrix calculated from their geographic coordinates using the Geographic Distance Matrix Generator, v.1.2.3 (Ersts, American Museum of Natural History, Center for Biodiversity and Conservation). Values of genetic variations were grouped within geographic distance classes (0-200, 201-400, 401-600, 601-800 km) and plotted as box plots in R v.2.15.3 (45). The correlation between genetic similarities and geographic distances was investigated using a Mantel test in Genealex 6.501 (46), and its significance was tested on 9.999 permutations.

Trapping experiment

The legume species *Dipogon lignosus* (L.) Verdc. (Phaseoleae), *Indigofera filifolia* Thunb. (tribe Indigofereae), *Macroptilium atropurpureum* (DC.) Urb. (siratro; tribe Phaseoleae), *Mimosa pudica* L. (tribe Mimoseae), *Podalyria calyptrata* C.A. Sm. (Podalyrieae) and *Psoralea pinnata* L. (Psoraleeae) were grown in soil samples from Malawi (Chinyonga, Blantyre - S15.819431, E35.041753) and South Africa (Table Mountain National Park - S33.952532, E18.456871). Both sites are part of natural ecosystems with no history of cultivation or rhizobial inoculation. At each locality, soils were sampled from the top 0-20 cm from at least three field sites and bulked to generate a composite sample for rhizobial isolation. Soil pH was determined from 4 g samples of sieved (1 mm mesh) mixed in 40 ml 1M KCl.

The Cape legume species *I. filifolia* and *P. calyptrata* are endemic to the Western Cape Province of South Africa. *Psoralea pinnata*, also endemic to the Fynbos, became naturalized and invasive in South Australia and New Zealand (47). *Dipogon lignosus* and *M. pudica*, which are native to South Africa and South America respectively, are also considered as

invasive (48,49). All legume species of the trapping experiment, except for *Ps. pinnata*, which is strictly associated with *Mesorhizobium* (alpha-class of Proteobacteria) (26), have been shown to form associations with *Burkholderia.* (26,49,50). Siratro is a widely used species known to be very promiscuous with regard to symbionts (51) and was proven previously to be effectively nodulated by *Burkholderia tuberum* (29,52).

Nodulation was assessed by growing germinated seedlings (three replicates) in 20 cm diameter plastic pots filled with acid-washed sterile sand and a layer of 200 g of soil (the layer of soil was omitted from negative control pots). Seeds were surface-sterilized in 4% (w/v) sodium hypochlorite for 10 min., rinsed in six changes of sterile water, soaked in boiled water and pre-germinated at room temperature on 1.5% (w/v) agar plates until root emergence. Pots were covered with a layer of nylon PA6 beads (Lomold group HQ, South Africa) and provided with a sterile watering tube to prevent cross-contamination. All plants were watered with sterile de-ionized water every two days. Nodules were harvested from seedlings after two months and rhizobia were isolated on YEMA as previously described.

Host range study

Seeds of legume species from the tribes Crotalarieae, Hypocalypteae, Indigofereae and Podalyrieae were used for this study. Seeds were surface-sterilized with concentrated sulphuric acid for 10 min. followed by 4% sodium hypochlorite for 10 min. Seedlings were grown in glass tubes with a sterile mixture of Vermiculite/Perlite as a rooting medium and fed with Jensens N-free plant nutrient medium under aseptic conditions (53). After one week of plant cultivation, seedlings were inoculated with the wild type strains *B. tuberum* STM678^T and *B. phymatum* STM815^T (54). Plants were harvested after 6 weeks and inspected for nodule formation and the potential ability to perform symbiotic nitrogen fixation was assessed by the presence of leghemoglobin (Lb). In addition, nodules were fixed and embedded for

light microscopy to assess their internal structure, as this is also a strong indicator of effectiveness (26,55). Three species of *Podalyria* and one *Virgilia* species, *V. oroboides* (tribe Podalyrieae), were also inoculated with a GFP-marked *B. tuberum* STM678 variant strain (29); nodule preparation and morphological observation of the STM678-GFP construct in nodule sections, using light and fluorescence microscopy, are according to (29). Uninoculated plants served as controls.

Nucleotide sequence accession numbers

The 16S rRNA sequences were deposited in the GenBank database under the accession numbers KF791602-KF791673 and KF824727-KF824733. The *recA* sequences were deposited under accession numbers KF791796-KF791864, KF824747-KF824753, KP013139-PK013158 and KT700208-KT700213. Sequences for the *nodA* sequences were deposited under the accession numbers KF791743-KF791795, KF824740-KF824746, KP013159-KP013178 and KT700202-KT700207. Sequences for the *nodC* sequences were deposited under the accession numbers KP013126-KP013137.

Results

16S rRNA *gene sequence cut-off levels used for (putative new) species delineation*

A large 16S rRNA dataset comprising 75 validly named *Burkholderia* species was constructed to evaluate the genetic diversity of *Burkholderia* at five different sequence similarity threshold values ranging from 97% to 99% (Fig. S1). A sequence similarity level to delineate the true number of sequences at species level was obtained between a cut-off value of 98.5% and 99%, resulting in 59 and 96 OTUs. Although there is some controversy about the concept of a species in prokaryotes (56-59) the results of the empirical clustering analysis, using 16S rRNA data, support 98.5% as a conservative threshold value for species level definitions within *Burkholderia* and corresponds to the general threshold value of 98.65% estimated to delineate the global prokaryotic diversity (60). A 98.5% threshold value was used for further diversity calculations of 16S rRNA datasets.

Phylogenetic clustering of the Burkholderia *richness according to geography and legume subfamily*

The diversity of root-nodulating *Burkholderia* was classified according to geography and their hosts for different DNA regions (16S rRNA, *nifH*, *nodA* and *nodC*). Table 1 shows the 16S rRNA OTUs and clusters of symbiosis genes calculated at a cut-off value of 98.5%, which are identified from different continents and host associations occurring across continents and legume subfamilies. From a total of 23 16S rRNA OTUs, eight groups occurred on more than one continent, including one OTU (number 5) globally distributed across all four continents assessed and three OTUs (numbers 1, 5 and 12) associated with both legume subfamilies (Table 1). *Burkholderia tuberum* (OTU number 1) was a highly recorded species (107 16S rRNA sequences) associated with eight South African genera (*Amphithalea*, *Aspalathus*, *Cyclopia*, *Hypocalyptus*, *Lebeckia*, *Podalyria*, *Rhynchosia* and

335 *Virgilia*) and from field nodules of the South American genus *Mimosa* (Table 1). Six OTUs
336 (numbers 4-8 and 15) comprised symbionts of invasive *Mimosa* species, recorded from South
337 America, as well as their invasive regions in Asia and Australia (Table S1).
338 In contrast to 16S rRNA, fewer nodulation and nitrogen fixation types were shared among
339 continents, including only four *nifH* (numbers 2, 4, 5 and 9), five *nodA* (numbers 3, 5, 6, 10
340 and 16) and three *nodC* (numbers 4, 9 and 10) types. One group of *nodC* (type number 4) and
341 one group of *nifH* (type number 4) sequences were globally distributed on all the four
342 continents. A total of five sequence clusters were shared between both subfamilies for *nifH*
343 (numbers 1 and 4), *nodA* (numbers 3 and 14) and *nodC* (number 4). All *nodA* and *nodC*
344 sequence clusters associated with both legume subfamilies originate from mimosoids and
345 from the papilionoid hosts *Macroptilium* and *Phaseolus.*
346 Phylogenetic NeighborNet analyses for chromosomal (16S rRNA), nitrogen fixation (*nifH*),
347 and nodulation (*nodA* and *nodC*) genes revealed the genetic divergence and clustering among
348 sequence types and their affinities for a geographical locality and legume subfamily (Fig. 2).
349 The genetic distances, proportional to evolutionary divergences, were more pronounced for
350 the symbiosis genes (*nifH*, *nodA* and *nodC*) than for the conservative 16S rRNA gene. For
351 16S rRNA, phylogenetic relationships among OTUs were not structured by geography nor
352 host (Fig. 2A). Large genetic clusters contained OTUs from different continents and
353 subfamilies, confirming the previous observation of shared 16S rRNA types across localities
354 and hosts (Table 1). In contrast to 16S rRNA, NeighborNet analyses of nitrogen fixation
355 (*nifH*) (Fig. 2B) and nodulation (*nodA* and *nodC*) genes (Fig. 2C-D) identified a strong
356 pattern according to geography and host. Genetic clusters were identified, separating the
357 African papilionoids from the South American and Asian mimosoid representatives.
358

359 Burkholderia *diversity, specificity and geographical distribution in legumes of the Fynbos*
360 *biome*

361 In order to investigate the biodiversity and geographic distribution of *Burkholderia* at a
362 smaller spatial scale, rhizobia of diverse indigenous Fynbos species were sampled and
363 analyzed using a combination of culture and non-culture based identification techniques.
364 Initially, a standard culture method was applied to selected legume lineages covering most
365 legume groups (Table S2; *Amphithalea, Aspalathus, Bolusafra, Crotalaria, Dipogon,*
366 *Hypocalyptus, Indigofera, Podalyria, Rafnia, Rhynchosia* and *Virgilia*) and all rhizobia were
367 identified as *Burkholderia*, showing only a single colony morphotype in each root nodule.
368 All cultured strains were authenticated using siratro (Table S2), showing effective nodules
369 and enhanced plant growth compared with nodule-free controls. Only the strain from *Rafnia*
370 *acuminata* (Dlodlo 22) failed to form effective nodules on siratro and so was not regarded as
371 a rhizobial symbiont.

372 In addition, a culture independent approach was performed using direct PCR analyses to
373 assess the nodule rhizobial diversity and to confirm single strain occupation within a nodule.
374 PCR amplifications on the total genomic DNA extraction of the intranodular tissue produced
375 high quality and single-copy sequences for all genetic markers investigated, suggesting one
376 dominant *Burkholderia* strain as nodule resident. Amplified 16S rRNA products were cloned
377 for available nodules in selected species within genera of two legume tribes (*Podalyria*:
378 Muasya, 6490; 6463 and *Indigofera*: Muasya & Stirton, 6502B; 6502C) to test the one-
379 symbiont one-nodule specificity. For all samples investigated, similar 16S rRNA clones (20
380 per sample) were obtained showing a single bacterial endosymbiont in each nodule.

381 Sequence data of 16S rRNA, *recA* and *nodA* from rhizobia of 26 Podalyrieae (13 individuals,
382 3 genera), 11 Indigofereae (8 individuals, 1 genus), 4 Hypocalypteae (2 individuals, 1 genus),
383 15 Crotalarieae (6 individuals, 3 genera) and 16 Phaseoleae (9 individuals, 3 genera) were

analyzed with Maximum Likelihood and Bayesian phylogenetic analyses (Figs. 3-4), clustering the isolates within diverse reference strains, comprising root-nodulating (*B. dilworthii* WSM3556^T, *B. dipogonis* LMG19430^T, *B. kirstenboschensis* Kb15^T, *B. rhynchosiae* WSM3937^T, *B. sprentiae* WSM5005^T, *B. tuberum* STM678^T) and plant-beneficial (*B. phytofirmans* PsJN^T, *B. xenovorans* LB400^T) lineages. Our isolates from various host legumes (*e.g. Amphithalea*, *Aspalathus*, *Indigofera*, *Rafnia*, *Rhynchosia*, *Podalyria*) were closely related to nodulated representatives (*B. kirstenboschensis*, *B. rhynchosiae*, *B. tuberum*) of the current Fynbos record, but the majority of isolates appeared to be related to bacteria without generally nodulating traits (*B. phytofirmans*, *B. xenovorans*) or were grouped apart into clusters without known reference species (Fig. 3).

Analyses of rhizobial lineages in relation to their geographical provenance showed many widely distributed 16S rRNA OTU types, suggesting genetic similarity of *Burkholderia* in Fynbos soils. To evaluate the diversity of Fynbos rhizobia in relation to geography at a regional scale, we investigated spatial structuring by the common approach of isolation by distance (61), assuming that geographic distance and population genetic differentiation are expected to correlate positively because population connectivity occurs more frequently among adjacent habitats. For close and distantly located populations, genetic variation was examined among *Burkholderia* strains, showing no effect of geographical distance on the genetic distance for both *recA* and *nodA* sequence data (Fig. 5). Genetic differentiation was constant among the different distance classes (0-200; 201-400; 401-600; 601-800 km), showing mean values of genetic similarities of ca. 94% and 96% for *recA* and *nodA*, respectively (Fig. 5). A Mantel test examined the associations between pairwise differences in genetic and geographical distances, rejecting an effect of geographical distance on the genetic *Burkholderia* variation ($P > 0.05$).

Similar to geography, no link was observed between *Burkholderia* strains and host genotype. For the majority of hosts, different populations of one legume species were associated with a set of genetically diverse strains of *Burkholderia* for both chromosomal and nodulation data (Figs. 3-4). Sequence analyses showed that a given *Burkholderia* lineage was associated with different legume lineages and in turn these host plants accommodated genetically diverse symbionts.

Nodulation of Cape legumes in African soils and identity of rhizobial groups

Nodulation of the legumes *I. filifolia*, *P. calyptrata* and *Ps. pinnata*, which are restricted in distribution to the Cape Fynbos biome, and the widely distributed species *D. lignosus* and *M. pudica* was assessed in South African (Fynbos region) and Malawian (Savanna grassland) soils. The pH of the soil from the Fynbos (pH = 4.6 ± 0.2) was substantially lower than at the Savanna site (pH = 7.1 ± 0.3). Distinct symbiotic associations were found among the legumes with a strong influence of the source of soils on the rhizobia sampled (Figs. 6-7). *Podalyria calyptrata* (Podalyrieae), *I. filifolia* (Indigofereae) and *D. lignosus* (Phaseoleae) were exclusively nodulated by *Burkholderia* in Fynbos soil, with the exception of one *Bradyrhizobium* isolate associated with *D. lignosus* that was from Fynbos soil. None of these legume species nodulated in Malawian soil, except *Ps. pinnata* (Psoraleeae) and siratro (Phaseoleae) that were able to form nodules in both soils (Table S4), with isolates identified as *Mesorhizobium* (*Ps. pinnata* – Fynbos), *Burkholderia* (siratro – Fynbos) and *Bradyrhizobium* (*Ps. pinnata*, siratro – Malawi). *Mimosa pudica* formed no nodules in either the South African or the Malawian soils.

The *Burkholderia* and *Mesorhizobium* symbionts isolated from legumes growing in Fynbos soils were placed in different clades (Figs. 6-7) and were highly related (99-100% sequence similarity) to known reference strains previously isolated from various South African legumes (Table S4). The *recA* and *nodA* sequence data of bradyrhizobia symbionts from the

433 Malawian soils were related (97-99%) to known African, South American and European
434 isolates (Table S4).
435
436 *Host range of* Burkholderia tuberum *and* B. phymatum *among South African legumes*
437 The host range experiment showed that all legumes from the tribes Crotalarieae,
438 Hypocalypteae, Indigofereae and Podalyrieae were able to nodulate successfully with the
439 type strain of *B. tuberum*, STM678^{T}, except for four *Calpurnia* species, which either did not
440 produce nodules (*C. aurea* and *C. intrusa*) or showed ineffective nodulation (*C. glabrata*
441 and *C. sericea*) (Table 2; Fig. S2). All legume species assessed in the host range experiment
442 are native Fynbos species, except for *Calpurnia*, where only *C. intrusa* is found in the
443 karroid vegetation near the Fynbos-dominated Swartberg Mountains. The presence of *B.*
444 *tuberum* in the nodule structure was confirmed in *Podalyria* and *Virgilia* species by
445 fluorescence microscopy of the GFP transconjugant strain of STM678 (Fig. 8), and in all the
446 other species by immunogold labelling with a *Burkholderia*-specific antibody (Fig. S2). The
447 type strain of *Burkholderia phymatum*, STM815^{T}, formed functional nodules on four native
448 Fynbos legume species of the tribe Podalyrieae (*Cyclopia* and *Virgilia*), whereas other
449 species of the genera *Amphithalea* (tribe Podalyrieae), *Hypocalyptus* (tribe Hypocalypteae),
450 *Aspalathus* and *Lebeckia* (both tribe Crotalarieae) produced ineffective nodules or remained
451 nodule-free (Table 2; Fig. S2).
452

Discussion

Spatial distribution of root nodulated Burkholderia at continental scale

The global survey of the *Burkholderia* record revealed various geographical and host-related patterns within the 16S rRNA and *nifH*, *nodA* and *nodC* datasets at a continental scale. Chromosomal 16S rRNA types were highly diverse (Fig. 2A, Table 1) and unrelated to the host subfamily or geographical region, whereas nitrogen fixation and nodulation genes are clearly structured by a geographical and host factor (Fig. 2B-D) with only a few sequence groups identified across continents and legume subfamilies (Table 1). The observation of an association between geography, host legume and nodulation genes, showing two large clusters of highly diverged nodulation gene types, according to their geographical origin and host subfamily, corroborates previous *Burkholderia* studies (12,19). All African distributed rhizobia were clustered in one group, and were highly diverged (<75% gene similarity) from the remaining mimosoid-related *Burkholderia*.

The geographical distribution of the legume host seems to be the key factor, explaining the nodulation and nitrogen fixation gene phylogenetic structure at a continental scale, supporting the idea that the rhizobial biogeography largely follows their hosts (20), which represent two distinct legume floras of South African papilionoids and South American mimosoids in the Fynbos and Cerrado/Caatinga biomes, respectively (12,62). Evidence is accumulating that the vast majority of *Mimosa* species native to central Brazil are exclusively associated with *Burkholderia* (10,55), whereas in Mexico, which is considered as another large centre of radiation of the genus, most endemic species are not nodulated by beta-rhizobia (17), but are specifically associated with alpha-proteobacteria and only a few lineages are able to form interactions with *Burkholderia* (11,63). Distinct nodule occupancies of beta- and alpha-rhizobia within the native home range of Brazilian and Mexican *Mimosa* species, respectively, can be largely explained by a combination of geographical separation

of the various *Mimosa* clades with distinct symbiont preferences, and their subsequent co-evolution with rhizobia in contrasting soil types (*e.g.* acid versus neutral/alkaline soils) (11). Conversely, access and availability of rhizobia, due to varied adaptation to edaphic and climatic factors, may be a critical factor governing dispersal of legumes outside native areas and thereby influence legume biogeographic patterns. The latter may be true for South Africa and Western Australia, which have frequent angiosperm dispersal events in the Cenozoic (64), associated with similarity of niches (Mediterranean climate, oligotrophic acidic soils), yet legumes are one of the few (large) families that do not exhibit disjunction between the two continents. While the endemic Australian tribes Bossiaeeae and Mirbelieae are largely associated with *Bradyrhizobium* lineages (65,66), the tribe Hypocalypteae, which is endemic to South Africa and resolved as a sister group to the mirbelioids is strictly associated with *Burkholderia*.

The nodulation genes *nodA* and *nodC* are frequently used to understand the symbiotic specificities and their evolutionary adaptation to a specific host (67). Because nodulation genes are involved in the synthesis of Nod-factors (i.e. rhizobial signaling molecules required for the earliest host responses) they determine the host specificity (68-70) and have been frequently shown to align with their *Burkholderia* host (12,17,28). The specificity of the symbiotic association of *Burkholderia* with mimosoid and papilionoid legumes is clearly demonstrated in one single species, *B. tuberum*, which has distinct nodulation genes or symbiotic variants and has been ascribed to symbiovars mimosae and papilionoideae, respectively (71,72). However, a link between *nodA* types and the legume subfamily is not strictly predictable for all species. *Macroptilium atropurpureum* (siratro, Papilionoideae) for example, known as a valuable plant for trapping a broad range of alpha- and beta-rhizobia (52), is able to nodulate with both *B. tuberum* sv. papilionoideae (e.g. STM678^T) (29) and sv. mimosae strains (e.g. STM4801) (71). Similarly, the mimosoid symbiont *B. phymatum*

503 STM815^T has been isolated from nodules of the papilionoid *P. vulgaris*, which is known for
504 its wide range of symbiotic partners (31). Apart from the records involving promiscuous host
505 legumes (siratro, *P. vulgaris*), *Burkholderia* species and their nodulation genes appear to
506 group and evolve in close concert with their mimosoid and papilionoid hosts. However,
507 evidence is accumulating that, although rhizobial species (*e.g. B. tuberum* sv. papilionoideae)
508 associated with the subfamily of Papilionoideae appear incapable of nodulating mimosoid
509 hosts (29), the opposite is not the case (12). In addition to common bean (73), diverse
510 papilionoids such as the Fynbos species *Dipogon lignosus* (49) and legumes of the genera
511 *Cyclopia* and *Virgilia* (Table 2, Fig. S2) have been demonstrated to form effective nodules
512 with the mimosoid-nodulating *B. phymatum*-type symbiont (17, 74), confirming its broad
513 host range and ability to associate with legumes of the mimosoid and papilionoid subfamily.
514 While symbiosis genes are largely structured according to legume subfamily, 16S rRNA
515 clusters are more diverse (Fig. 2A), affiliated with various hosts from different parts of the
516 world (Table 1). A widespread occurrence of *Burkholderia* strains, especially for 16S rRNA
517 types (Table 1), indicates an inter-continental and global distribution pattern for different
518 strains of burkholderias (*e.g. B. diazotrophica, B. mimosarum, B. phymatum, B. sabiae* and
519 *B. tuberum*). The occurrence and vast diversity of *Burkholderia* outside Africa and South
520 America are mostly recorded from pan(sub)tropically distributed *Mimosa* species (*M.*
521 *pudica, M. pigra, M. diplotricha*). *Burkholderia* symbionts of these widespread invasive
522 plant species are included in the clustering analyses and close relationships of nodulation
523 genes with their native distributed relatives support previous observations that rhizobia are
524 co-transported with the seeds or plants from their native to new invasive habitats. Following
525 the co-introduction hypothesis (75), symbionts that have been co-introduced with their hosts
526 or which have hitchhiked on introduced material over long-distances, bridging geographical
527 barriers between continents, has been evidenced in many studies (15,49,76-78). For

Burkholderia, a plausible long-distance migration event from South Africa to New Zealand, possibly dispersed across the Australian continent, has been reported in the South African papilionoid *Dipogon lignosus* (tribe Phaseoleae) (79), which is invasive in New Zealand and Australia (49, 79) as revealed by high sequence similarities of the symbiosis genes (*nodA* sequence clusters 6, 10; *nodC* sequence clusters 9, 17) between invasive populations of *Dipogon* and native South African relatives from the genera *Bolusafra*, *Crotalaria*, *Cyclopia*, *Hypocalyptus*, *Indigofera*, *Podalyria* and *Rhynchosia*.

Geographical distribution and specificity of Fynbos Burkholderia

While the global *Burkholderia* diversity was structured for the nodulation genes at legume subfamily level, an interaction between rhizobia, host legumes and geographical distribution was not shown at regional scale, showing widely spread and locally diverse *Burkholderia* populations in the Fynbos. Our results corroborate a previous study, demonstrating the widespread occurrence of *Burkholderia* and the absence of a site sampling effect on the rhizobial diversity of selected Hypocalypteae and Podalyrieae species (24,30). Using geographical distances as a proxy for population connectivity, genetic variation is expected to correlate positively with the sampling site distances. Our study does not show any correlation between genetic variation and geographical distance, suggesting the absence of genetic isolation through high rates of rhizobial dispersal of both chromosomal and symbiosis traits.

In the Fynbos region, local environmental variables, rather than spatial dispersal factors, are most likely the major ecological drivers for rhizobial distributions. In a recent study, Lemaire and associates (26) showed that genetic variation of Fynbos *Burkholderia* was correlated with differences in site elevation, a feature also observed in symbionts of South American

552 *Mimosa* species (10); hence the indirect effects of temperature and rainfall may play a
553 significant role in the rhizobial community structure.
554 Symbiotic associations of Fynbos legumes for *Burkholderia* have been described in many
555 lineages with various degrees of specificity. In the tribe Podalyrieae, a strong preference for
556 *Burkholderia* is observed, showing all legume species and genera (except for *Calpurnia*
557 which is not endemic to the Fynbos – Table 2, Figure S2) strictly nodulated with
558 *Burkholderia* (12,24,26). Other common plant groups such as the tribes Crotalarieae and
559 Indigofereae also contain *Burkholderia*-philous species, although (closely related) legume
560 lineages within the same tribes and co-occurring in the similar habitats have been recorded
561 with classical alpha-rhizobial lineages ((26) and references therein).
562 In this study, the *Burkholderia*-legume interaction was further investigated at a finer
563 taxonomic scale. Diverse phylogenetic clusters of *Burkholderia* strains were observed within
564 native legume genera of the tribes Crotalarieae (*Aspalathus*, *Crotalaria*, *Lebeckia*, *Rafnia*),
565 Indigofereae (*Indigofera*), Phaseoleae (*Bolusafra*, *Dipogon*, *Rhynchosia*), Podalyrieae
566 (*Amphithalea*, *Podalyria*, *Virgilia*), but without a host specific pattern (Figs. 3-4). For both
567 chromosomal and nodulation genes, the latter symbiotic genes determining host specificity
568 (68), a relaxed association among genetically similar rhizobia and different legume species,
569 genera and tribes was demonstrated. The variation of host-*Burkholderia* interactions
570 corroborates a previous rhizobial screening in selected legume genera of the tribes
571 Hypocalypteae (*Hypocalyptus*) and Podalyrieae (*Cyclopia*, *Podalyria*, *Virgilia*) (24,30). In
572 South America, a similar relaxed host specific interaction has been described for
573 *Burkholderia* and their mimosoid hosts (10,62). The predominance or prevalence of
574 *Burkholderia* strains in both papilionoid and mimosoid legumes, but without a host specific
575 pattern, indicates that the host genotype has not been a major factor on the local *Burkholderia*
576 distribution. This observation is in line with the current host range study, showing selected

South African papilionoid species able to form effective nodules with the strains *Burkholderia tuberum* STM678^T and *Burkholderia phymatum* STM815^T. Strains of *B. phymatum*, which is found as a common symbiont of *Mimosa* in French Guiana, Papua New Guinea, India and China (12,16,17,71), has not been isolated from field nodules collected in the Fynbos, yet they are able to nodulate selected papilionoids (*Dipogon*, *Cyclopia*, *Virgilia*). The promiscuous character of the papilionoid-*Burkholderia* symbiosis has previously been demonstrated in other species of Podalyrieae (12) and Phaseoleae (29,49,52).

Although Fynbos legumes were generally associated with diverse *Burkholderia* species, individual root nodules consistently accommodated a single strain. The observation of a single *Burkholderia* strain per nodule may suggest high selective constraints of the host towards their symbiont. In order to retain a stable and mutualistic interaction, legumes generally hinder the emergence of opportunistic rhizobial strains and select cooperative (i.e. effectively nitrogen-fixing rhizobia) ones over non-beneficial symbionts (referred to as partner choice) (80,81) by providing only one beneficial symbiont with ample carbon resources while an uncooperative nodule occupant is disfavored with host resources (referred to as host sanctions) (82,83). However, the general observation of a relaxed interaction or accommodation of diverse rhizobial strains per host individual may indicate that the one-nodule one-strain interaction is a result of high competitiveness for nodulation among rhizobial strains, rather than to selection by the host plant.

Nodulation of Fynbos legumes outside their distribution range

A legume growing in non-native soil can only form nodules when naturalized populations of compatible rhizobia are available in the soil. In our inoculation experiment, siratro and *Ps. pinnata* nodulated in soils collected from South Africa and Malawi, whereas *P. calyptrata*, *I. filifolia* and *D. lignosus* were nodule-free in the Malawian soil. The inability to form nodules

602 in Malawian soil suggests that these legumes, known to exhibit a strong host preference for
603 *Burkholderia* (24,26,84), did not find their specific *Burkholderia* symbionts in the Malawian
604 (Savanna) soil, which was substantially higher in pH compared to the Cape soil. The
605 occurrence and success of *Burkholderia* in South African (Fynbos) soils, but also in the
606 South American Cerrado/Caatinga biomes, can be linked with the general ecological
607 adaptation of these symbionts to acidic soil conditions, which may play a prominent role as
608 ecological driver on the rhizobial diversity (19,27,28,32). In Malawi, legume nodulation by
609 *Burkholderia* has never been reported as far as we know, and further *Burkholderia* surveys in
610 other African soils are needed to provide evidence for a more limited distribution pattern on
611 the African continent with the Fynbos biome reported as a major center of diversity.
612 The inability of legumes to form a symbiosis with *Burkholderia* in Malawian soils does not
613 necessarily indicate the absence of *Burkholderia* in other regions of Africa (*e.g.* see report of
614 *Burkholderia* nodulating the non-native common bean in Moroccan soil (31)), but may also
615 result from incompatible types of symbiosis genes within local *Burkholderia* communities. In
616 this context, the observation that *Mimosa pudica* is unable to nodulate within the
617 *Burkholderia*-rich Fynbos soils, strongly suggests that the necessary mimosoid type
618 nodulation genes (which are genetically distinct from the papilionoid type nodulation genes)
619 are not naturally occurring in these soils. The absence of effective rhizobia and their
620 compatible symbiosis genes is a potential barrier to the colonization of novel habitats by the
621 host legumes. For exotic legumes such as *Mimosa pudica*, it appears that the host needs to
622 bring its own native symbionts into the new environment for an optimal and successful
623 colonization and distribution (15,16).
624 In contrast to legumes with a specific preference for *Burkholderia*, *Ps. pinnata* was nodulated
625 by *Mesorhizobium* in Fynbos soils and by *Bradyrhizobium* in Malawian soil, indicating a
626 more relaxed interaction, albeit one that does not involve beta-rhizobia. Although field

nodules of this genus have been consistently associated with *Mesorhizobium* in the Fynbos (26), *Bradyrhizobium* was also able to nodulate *Psoralea* effectively, probably in the absence of their preferred *Mesorhizobium* symbionts in these Savanna soils. The genus *Psoralea* has a centre of diversity in the Fynbos but several species occur in montane grasslands in North-Eastern South Africa, Mozambique and Swaziland, and two species are naturalized in Australia (47). The current *Mesorhizobium* diversity from Fynbos *Psoralea* has been placed in a separate cluster unrelated to known 16S rRNA or *nodA* gene types from other African localities, suggesting rhizobial strains restricted to the Cape region. The *Bradyrhizobium* isolates from the Malawian soils, however, were closely related to *B. elkanii*, and are geographically widespread and able to nodulate a broad range of legumes from different continents (65,85-88). In a recent study by Parker (89), a phylogenetic analysis on a broad sampling of *Bradyrhizobium* strains from diverse plant groups provided evidence for a broad host range of most bradyrhizobia lineages, including *B. elkanii*, that are associated with diverse legume tribes.

Concluding remarks

Burkholderia populations, like many free-living microbes and other (classical) rhizobial groups, are widespread and occur on different continents (except Antarctica and Europe), a phenomenon which can be explained by their capacity for long-distance dispersal. By investigating nodulation genes of publicly available sequence data, rather than taxonomic identities (16S rRNA types), we observed a strong biogeographic relationship, which corresponds largely to two main groups of *Burkholderia* with distinct host related affinities. Indeed, various phylogenetic studies have described taxonomically diverse papilionoid- and mimosoid-associated rhizobia with a geographical structure preserved in the nodulation genes (*nodA* and *nodC*), supporting the hypothesis that traits (i.e. nodulation genes) rather

652 than taxon names (i.e. chromosomal genes) are the fundamental units of biogeography (90).
653 In contrast to the global investigation of *Burkholderia*, regionally distributed species in the
654 Fynbos did not show any geographical distribution pattern. Within the Cape region, genetic
655 variation for both chromosomal and nodulation genes was unrelated to geographical or host
656 factors, suggesting that nodulating *Burkholderia* are omnipresent in the Fynbos biome and do
657 not constrain the distribution of their native host legumes in terms of compatible symbionts.
658

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Figure Legends

Table 1.

Occurrence of 16S rRNA OTUs and sequence clusters of symbiosis genes (*nifH*, *nodA* and *nodC*) shared among different continents (South America - SAM, Africa - AFR, Australasia - AUS and Asia - ASI) and host subfamilies (Mimosoideae - MIM and Papilionoideae - PAP). The host genera and reference strains of *Burkholderia* are listed per group (98.5% sequence similarity threshold value). - = not present

[1] *Burkholderia phymatum* STM815^T was allegedly isolated from the papilionoid *Machaerium lunatum* in French Guiana but has never been proven to renodulate its original host (12) or an alternative *Machaerium* species (*M. brasilense*, (17)).

16S rRNA OTU	Geographic Distribution	Host Subfamily	Reference strain	Host Genera of Mimosoideae	Host genera of Papilionoideae
1	SAM-AFR	MIM-PAP	*B. tuberum*	*Mimosa*	*Amphithalea, Aspalathus, Cyclopia, Hypocalyptus, Lebeckia, Macroptilium, Podalyria, Rhynchosia, Virgilia*
3	AFR-AUS	PAP	*B. dipogonis*	-	*Bolusafra, Crotalaria, Cyclopia, Dipogon, Hypocalyptus, Podalyria, Rafnia, Virgilia*
4	SAM-ASI-AUS	MIM	*B. mimosarum*	*Mimosa*	-
5	SAM-AUS-ASI-AFR	MIM-PAP	*B. phymatum*	*Mimosa, Parapiptadenia, Piptadenia*	*Machaerium*[1], *Phaseolus*
6	SAM-ASI	MIM	*B. sabiae*	*Abarema, Mimosa, Parapiptadenia*	-
7	SAM-AUS	MIM	*B. diazotrophica*	*Mimosa, Piptadenia, Anadenanthera*	-
8	SAM-AUS	MIM	-	*Mimosa*	-
12	AFR-SAM	MIM-PAP	-	*Mimosa*	*Hypocalyptus*

Sequence cluster *nifH* gene					
1	SAM	MIM-PAP	*B. tuberum*	*Mimosa*	*Macroptilium*
2	AFR-AUS	PAP	*B. tuberum*	-	*Amphithalea, Aspalathus, Crotalaria, Cyclopia, Dipogon, Hypocalyptus, Indigofera, Lebeckia, Podalyria, Rafnia, Virgilia*
4	SAM-AUS-ASI-AFR	MIM-PAP	*B. phymatum/B. diazotrophica*	*Abarema, Anadenanthera, Mimosa*	*Machaerium*[1]*, Phaseolus*
5	SAM-ASI-AUS	MIM	*B. mimosarum*	*Mimosa*	-
9	AFR-AUS	PAP	*B. rhynchosiae*	-	*Dipogon, Rhynchosia*
Sequence cluster *nodA* gene					
3	SAM-ASI-AUS	MIM-PAP[1]	*B. phymatum*	*Mimosa*	*Machaerium*[1]
5	SAM-ASI-AUS	MIM	*B. mimosarum*	*Mimosa*	-
6	AFR-AUS	PAP	*B. dipogonis*	-	*Crotalaria, Cyclopia, Dipogon, Hypocalyptus, Virgilia, Podalyria*
10	AFR-AUS	PAP	*B. rhynchosiae*	-	*Bolusafra, Dipogon, Indigofera, Rhynchosia*
14	SAM	MIM-PAP	*B. tuberum*	*Mimosa*	*Macroptilium*
16	SAM-ASI	MIM	*B. sabiae*	*Mimosa*	-
Sequence cluster *nodC* gene					
4	SAM-AUS-ASI-AFR	MIM-PAP	*B. phymatum/B. diazotrophica*	*Anadenanthera, Mimosa*	*Phaseolus*
9	AFR-AUS	PAP	*B. dipogonis*	-	*Crotalaria, Dipogon*
10	SAM-ASI	MIM	*B. mimosarum*	*Mimosa*	-

Table 2.
Nodulation of selected Fynbos species after inoculation with *Burkholderia tuberum* STM678^T or *B. phymatum* STM815^T. E = effective nodulation; I = ineffective nodulation, considered if inoculated plants are not greener than uninoculated controls and only few and white nodules are visible; - = not tested. New reports of nodulation are indicated in bold.

[1]Nodules tested with both *Burkholderia tuberum* STM678^T and STM678GFP.

*Data from Elliott *et al*. (29)

Tribe	Legume species tested	*Burkholderia tuberum* STM678^T	*Burkholderia phymatum* STM815^T
Crotalarieae			
	Aspalathus carnosa Bergius	E	no nodules
	Lebeckia ambigua E.Mey.	E	no nodules
Hypocalypteae			
	Hypocalyptus coluteoides (Lam.) R.Dahlgren	E	-
	Hypocalyptus sophoroides (P.J.Bergius) Baill.	E	I
Indigofereae			
	Indigofera filifolia Thunb.	E	-
Podalyrieae			
	Amphithalea ericifolia (L.) Eckl. & Zeyh	E	I
	Calpurnia aurea (Aiton) Benth.	no nodules	-
	***Calpurnia glabrata* Brummitt**	**I**	-
	Calpurnia intrusa (W.T.Aiton) E.Mey.	no nodules	-
	***Calpurnia sericea* Harv.**	**I**	-

Cyclopia subternata Vogel	E	E
Cyclopia genistoides (L.) Vent.	E*	E
Cyclopia intermedia E.Mey.	E*	E
***Liparia laevigata* Thunb.**	**E**	-
Liparia splendens (Burm.f.) Bos & de Wit	E	-
Podalyria burchellii DC.	E	-
Podalyria calyptrata (Retz.) Willd.	E[1]	-
Podalyria canescens E.Mey.	E[1]	-
***Podalyria leipoldtii* L.Bolus**	**E**	-
Podalyria myrtillifolia Willd.	E[1]	-
***Podalyria rotundifolia* (P.J.Bergius) A.L.Schutte**	**E**	-
Podalyria sericea R.Br	E	-
Stirtonanthus taylorianus (L.Bolus) B.-E.van Wyk & A.L.Schutte	E	-
Virgilia oroboides (P.J.Bergius) T.M.Salter	E[1]	E
***Xiphotheca fruticosa* (L.) A.L.Schutte & B.-E.van Wyk**	**E**	-

Figure 1
Map of South Africa showing the geographical distribution of sampling sites within the Western and Eastern Cape Provinces. Records of our isolates are indicated with white squares, whereas samples from other studies are shown with black dots.

Figure 2
NeighborNet networks of (A) 16S rRNA, (B) *nifH*, (C) *nodA* and (D) *nodC* sequence types. Sequence types exclusively recorded from one continent are shown by colored circles (Africa – green circles, South America – red circles, Asia – blue circles, Australasia – yellow circles). Numbers of sequence clusters sharing isolates from different continents and/or legume subfamily are shown in grey squares as listed in Table 1. Bootstrap support values below and above 50% are shown with grey and black branches, respectively. Scale bar represents substitutions per site.

Figure 3
Phylogenetic tree of rhizobial isolates of the Fynbos biome based on 16S rRNA and *recA* data. Support values for the Bayesian and Maximum Likelihood analyses are given at the nodes (Bayesian posterior probabilities – bootstrap support values for the Maximum Likelihood analysis). Reference strains are shown in bold.

Figure 4
Phylogenetic tree of rhizobial endosymbionts based on *nodA* data. Support values for the Bayesian and Maximum Likelihood analyses are given at the nodes (Bayesian posterior probabilities – bootstrap support values for the Maximum Likelihood analysis). Reference strains are shown in bold.

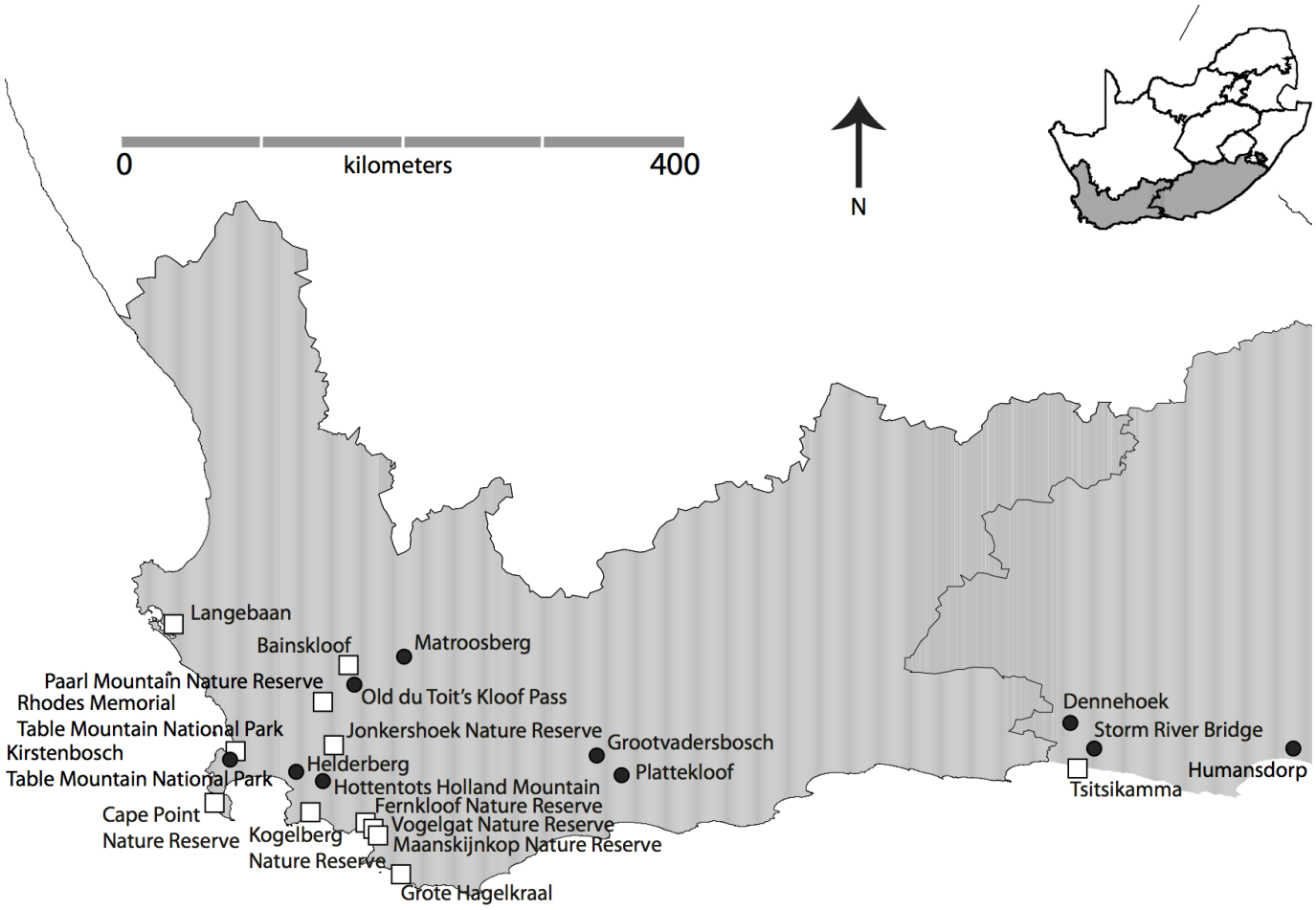
Figure 5
Box plots of pairwise genetic distances for (A) *recA* and (B) *nodA* sequence data grouped within four spatial distance classes (0-200; 201-400; 401-600-601-800 km). Box plots represent observations within 95% confidence intervals and the whiskers

extend from the box to the highest and lowest values, excluding outliers, which are shown as circles. The line across the box indicates the median.

Figure 6
Phylogenetic tree based on *recA* sequences of rhizobial isolates sampled from the trapping experiments. The closest reference strains obtained from BLASTN searches (see Table S4) are included in the analyses. Bayesian support values are given at the nodes. Geographic distribution of the isolates and reference strains are shown for each taxon. Number of substitutions per site is shown on the phylogram.

Figure 7
Phylogenetic tree based on *nodA* sequences of rhizobial isolates sampled from the trapping experiments. The closest reference strains obtained from BLASTN searches (see Table S4) are included in the analyses. Bayesian support values are given at the nodes. Geographic distribution of the isolates and reference strains are shown for each taxon. Number of substitutions per site is shown on the phylogram.

Figure 8
Fluorescence (A,C,E,F) and normal transmitted light (B, D) microscopy of sections (50 µm) from nodules of *Podalyria calyptrata* (A-B), *P. canescens* (C, D), *P. myrtillifolia* (E) and *Virgilia oroboides* (F) showing infected cells containing symbiotic bacteroids (*) as either green fluorescent (A, C, E, F) or dense opaque (B, D) regions in the nodule center. The green-yellow colour in the nodule cortex (A, C, E, F) results from autofluorescence of lignin and suberin. Bars = 100 µm.



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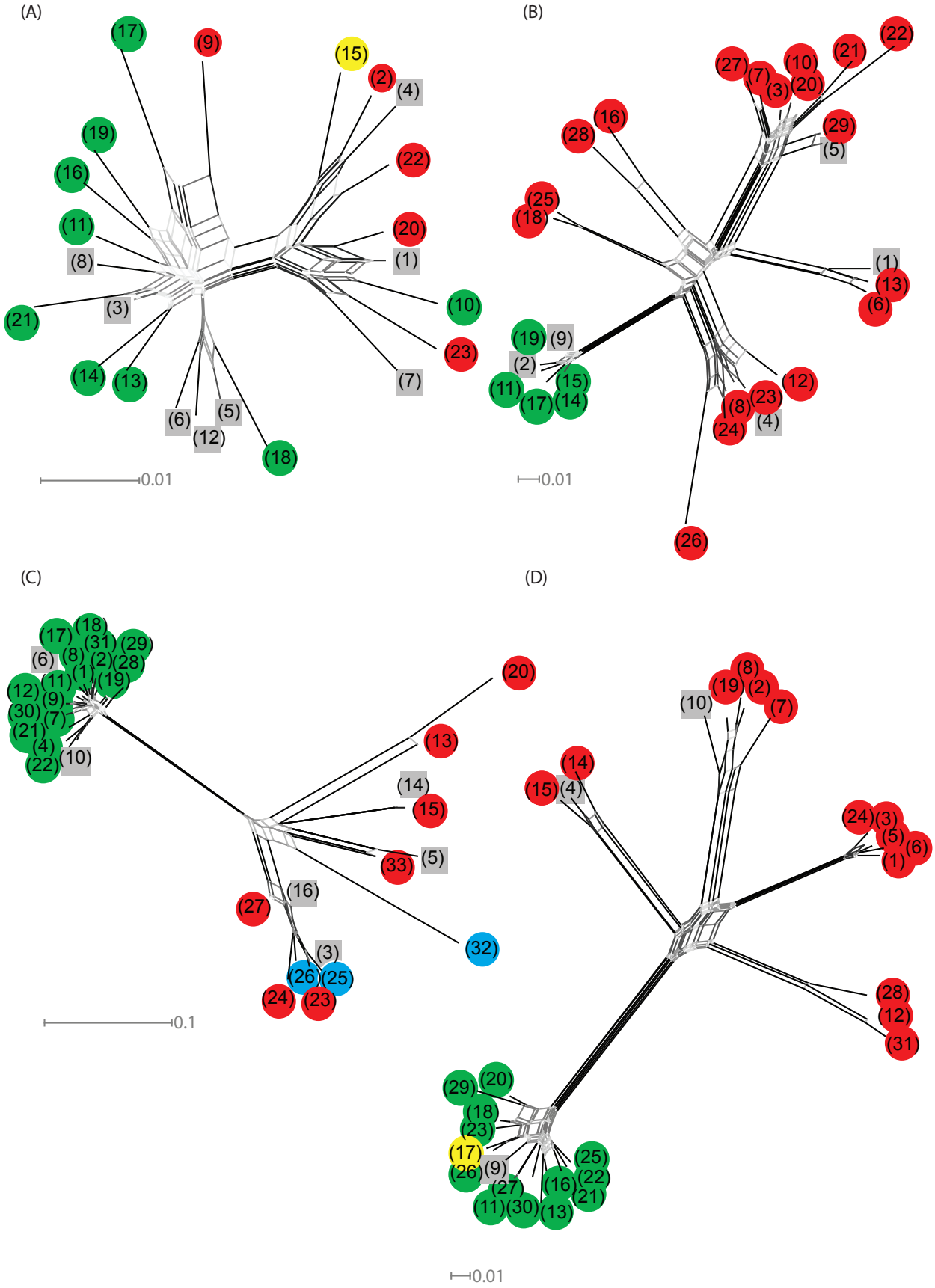

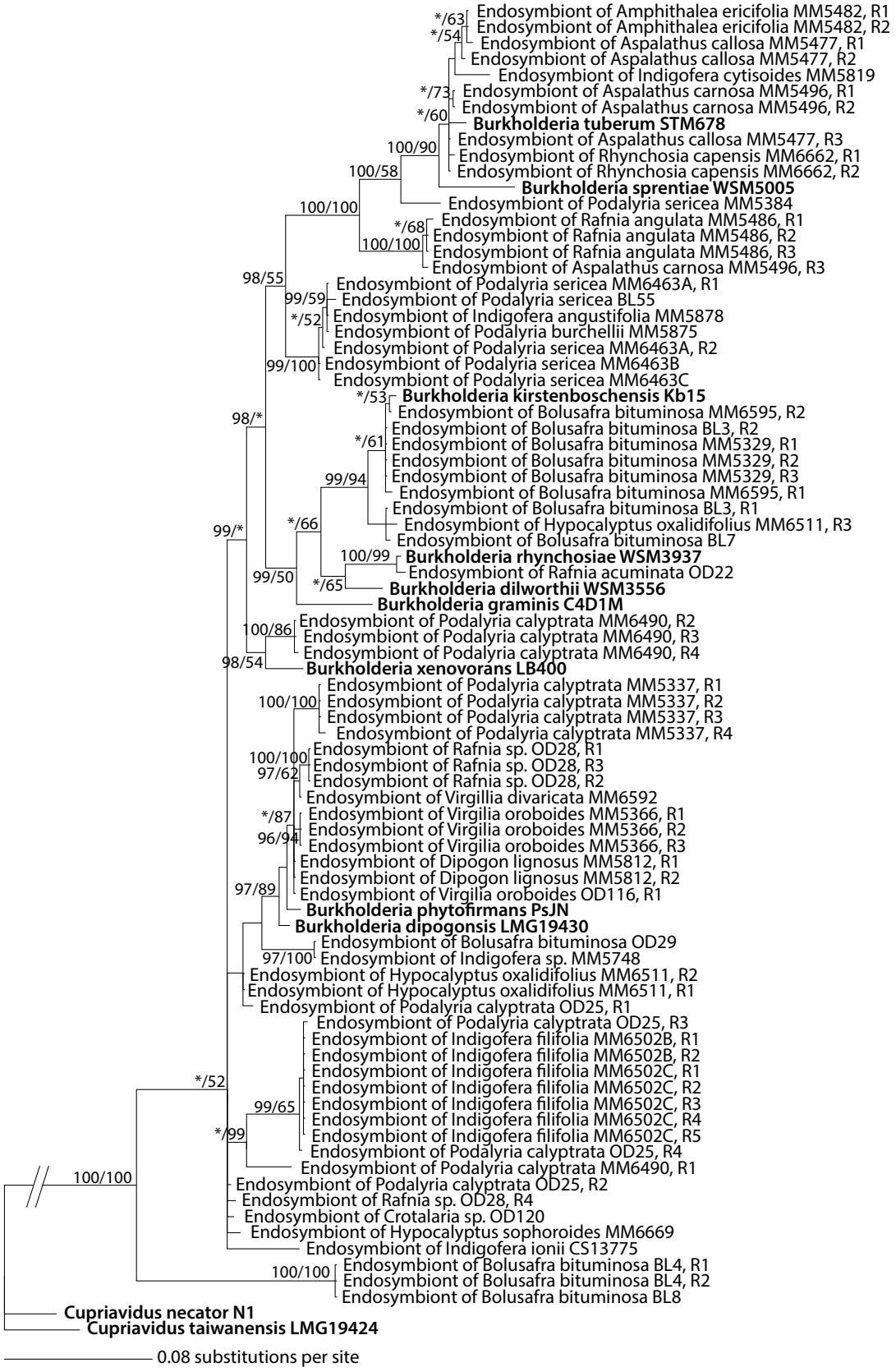

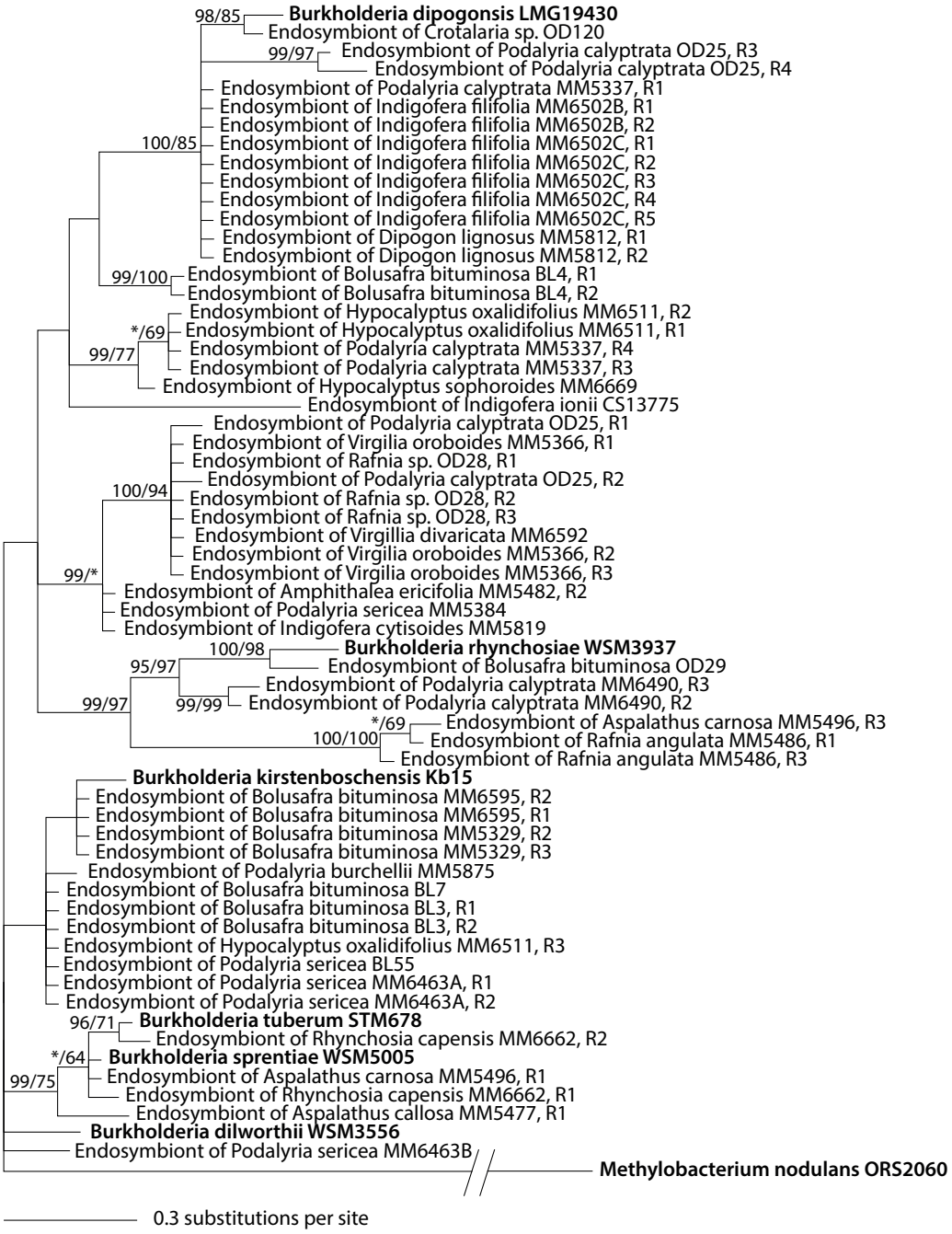

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